Original Paper

A Genetic Polymorphism (rs17251221) in the Calcium-Sensing Receptor is Associated with Breast Cancer Susceptibility and Prognosis

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Key Words

Calcium-sensing receptor • Breast cancer • Susceptibility • Prognosis

Abstract

Background: Calcium-sensing receptor (CaSR) is a typical G protein coupled receptor. The rs17251221 SNP is located in an intron of the CaSR gene, and the G allele is considered a gain of function mutation. Previous studies revealed that rs17251221 polymorphisms contribute to the risk of developing certain types of cancers. This study investigated the rs17251221 SNP in breast cancer by analyzing the correlation of the rs17251221 genotype with breast cancer susceptibility, clinicopathological features and prognosis. **Methods:** A TaqMan assay was used to genotype the rs17251221 SNP in a case-control study. The expression levels of CaSR in breast cancer tissues were determined using quantitative reverse-transcription PCR (qRT-PCR) and western blot analysis. The association of the rs17251221 genotype and the clinicopathological characteristics, as well as the prognosis of the breast cancer patient, was assessed statistically. **Results:** We found that the AG and GG genotypes were associated with lower mRNA and protein levels of CaSR compared to the AA genotype in breast cancer tissues. We also found that the AG and GG genotypes were associated with breast cancer susceptibility, the patient's age at diagnosis, tumor size, lymph node metastasis and estrogen receptor status of breast cancer tissue. More importantly, we found that the genotypes were prognostic markers for both disease-free survival and overall survival of breast cancer. **Conclusion:** The rs17251221 SNP is a risk factor associated with breast cancer susceptibility, as well as a prognostic indicator. Our data suggest that rs17251221 may be a potential therapeutic target in breast cancer.

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Introduction

Breast cancer is a major public health problem worldwide. Though the mortality rate of breast cancer has decreased due to improvements in early detection and/or treatment, approximately 234,580 new breast cancer cases are expected to be diagnosed in 2013, and approximately 40,030 patients will die from breast cancer [1]. Breast cancer is a heterogeneous disease that is influenced by both genetic and environmental factors. Genetic single nucleotide polymorphisms (SNPs) are important indicators of susceptibility to breast cancer [2, 3]. Studies have confirmed that the existence of genetic mutations, such as those in the BRCA1 and BRCA2 genes, are highly associated with breast cancer risk [4, 5]. Therefore, identification of additional potential markers may result in the increased prediction of breast cancer susceptibility and allow for the earlier application of appropriate therapeutic strategies to reduce mortality.

Calcium-sensing receptor (CaSR) was first cloned from the bovine parathyroid gland by Brown and his colleagues in 1993 [6]. Subsequently, the gene was mapped to chromosome 3q13 [7]. CaSR is a typical G protein coupled receptor with an extracellular domain, a 7 membrane spanning domain and an unusually long intracellular carboxyl terminal tail. The receptor is glycosylated in the endoplasmic reticulum, modified in the Golgi and is finally localized to the plasma membrane [8-10]. CaSR plays an important role in calcium homeostasis through the regulation of parathyroid hormone secretion and calcium reabsorption [8]. The rs17251221 SNP is located in an intron of the CaSR gene, and the G allele is considered a gain of function mutation. Genome wide meta-analysis revealed that the rs17251221 SNP was strongly associated with serum calcium regulation in individuals of European and Indian-Asian descent [11]. A previous study found that an inactivating mutation of CaSR (rs17251221) caused familial hypocalciuric hypercalcemia, which results in life-long hypercalcemia and leads to asymptomatic life-long hypercalcemia [12]. Chou et al. [13] revealed that the rs17251221 SNP was a susceptibility marker for stone multiplicity in nephrolithiasis. Recently, it was reported that the rs17251221 SNP was strongly associated with prostate cancer [14]. However, the genetic effect of CaSR (rs17251221) on the susceptibility and prognosis of breast cancer is still unclear. In this manuscript, we evaluated the association of the rs17251221 polymorphism with breast cancer susceptibility, clinicopathological variables and prognosis.

Patients and Methods

Patients and samples

This study involved 217 breast cancer patients who were diagnosed at Qilu Hospital of Shandong University between 2001 and 2011. All of the diagnoses were made by at least two pathologists according to the World Health Organization Classification of Tumors. To detect the relationship between the rs17251221 polymorphism and breast cancer susceptibility, 231 age-matched females were recruited from the annual physical examination peoples. To analyze the association between the rs17251221 SNP and patient prognosis, 65 patients with long-term clinical follow-up were enrolled. Written, informed consent was obtained from all participants in this study, and the study was approved by the Ethical Committee of Shandong University.

DNA extraction

DNA was extracted from whole blood samples from both the patient and control groups using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The concentration and purity of the DNA were measured using an ultraviolet spectrophotometer (GE Healthcare, Piscataway, NJ, USA). The DNA samples were stored at -20°C until further use.

SNP genotyping analysis

TaqMan allelic discrimination analyses were performed according to the Applied Biosystems standard protocols (Applied Biosystems, Carlsbad, CA, USA). The TaqMan probes were synthesized by Takara (Dalian,

China). The genotyping assay was performed using a StepOnePlus Real-Time PCR System. The following amplification conditions were used: 95°C for 10 min, 45 cycles of 92°C for 15 s, 60°C for 60 s and 60°C for 30 s. To confirm our genotyping results, several DNA samples were randomly amplified and sequenced using polymerase chain reaction (PCR) analysis. The following sequencing primers were designed: forward primer 5'-GCCAATGGAGCAGCGTTCACAGA-3' and reverse primer 5'-ATCCTGCCTCAG CCTCCCAAAGT -3'.

Quantitative reverse-transcription PCR (qRT-PCR) analysis

Total RNA was extracted using the TRIZOL reagent, and cDNA was synthesized from 1 µg of total RNA using the PrimerScript RT Reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed using the SYBR green PCR mix on a StepOnePlus Real-Time PCR System. The following primer sequences were used: 5'-AAGGGGGACATTATCCTTGG-3' and 5'-GCTGGGCTGCTGTTTATCTC-3'. The samples were loaded in quadruplicate, and the results of each sample were normalized to β-actin. Each experiment was repeated at least three times to confirm the results.

Immunohistochemistry (IHC)

To evaluate the association between the rs17251221 polymorphism and the clinicopathological parameters, tissue microarrays were stained using the streptavidin-peroxidase-biotin (SP) method, as previously described [15, 16]. *CaSR* immunoreactivity was localized to the cytoplasm and was semi-quantitatively scored based on the percentage of positive cells. *CaSR* immunoreactivity was assigned one of the two final scores: negative (≤ 10% positive tumor cells) or (positive > 50% positive tumor cells). The tumor was scored positive for estrogen receptor (ER) and progesterone receptor (PR) when ≥10% of the tumor cells on the slide were positive for the receptor, regardless of the staining intensity. Human epidermal growth factor receptor 2 (HER2) status was evaluated according to the DAKO scores.

Statistical analysis

The allele frequencies were tested for Hardy-Weinberg equilibrium using the chi-square test [17]. A P-value >0.05 did not deviate from the equilibrium. The SPSS Statistics 18.0 (SPSS Inc. Chicago, Illinois, USA) software was used to analyze the data in our study. Unconditional logistic regression models were applied to analyze the relationship between the rs17251221 SNP and breast cancer susceptibility, as well as clinicopathological characteristics. Differences in patient survival were determined using the Kaplan-Meier method. The Cox hazard regression model was used to analyze the independent prognostic value of the rs17251221 SNP. A P-value <0.05 was considered statistically significant.

Results

The association between the rs17251221 polymorphism and its expression in breast cancer tissues

To explore the possible functions of the rs17251221 polymorphism in breast cancer, we first examined the mRNA levels of *CaSR* in 25 cases (16 cases of AA, 7 cases of AG, 2 case of GG) of breast cancer tissues, according to the different SNP genotypes. Next, we determined the mRNA levels of *CaSR* in these samples using quantitative reverse-transcription PCR analysis. As shown in Figure 1A and B, the average mRNA levels of *CaSR* were 3.495 ± 0.403 for the GG genotype, 3.087 ± 0.906 for the AG genotype and 1.023 ± 0.347 for the AA genotype. No significant difference was observed between the GG and AG genotypes ($p=0.575$). The GG and AG genotypes exhibited lower levels of *CaSR* than the AA genotype (both $p<0.001$). We also detected the levels of *CaSR* protein in tissues with the different genotypes and found that the AG and GG genotypes expressed decreased levels of *CaSR* compared to the AA genotype (Fig. 2). Taken together, the G allele was associated with relatively lower levels of both *CaSR* mRNA and protein expression in breast cancer tissues. This suggested that the rs17251221 polymorphism maybe a functional SNP in breast cancer.

The association between the rs17251221 polymorphism and breast cancer susceptibility

To investigate whether the rs17251221 polymorphisms is a susceptibility risk factor for breast cancer, 231 healthy women and 217 breast cancer patients were enrolled in this

Fig. 1. The relationship between the rs17251221 polymorphism and CaSR mRNA expression in breast cancer tissues. (A) The mRNA levels of CaSR in breast cancer tissues with the AA, AG and GG genotypes. (B) The mRNA levels of CaSR in breast cancer tissues with the AA and AG+GG genotypes.

Fig. 2. The protein levels of CaSR in breast cancer tissues with the AA, AG and GG genotypes.

Table 1. Genotype distribution in cancer cases and controls. OR, odds ratio; CI, confidence interval

	Number (%)		P value	OR (95%CI)
	Control (n=231)	Cancer (n=217)		
Genotypes				
AA	196(84.85%)	161(74.19%)		
AG	34(14.72%)	47(21.66%)	0.037	1.683(1.033-2.742)
GG	1(0.43%)	9(4.15%)	0.007	10.957(1.374-87.393)
AA	196(84.85%)	161(74.19%)		
AG+GG	35(15.15%)	56(25.81%)	0.007	1.948(1.216-3.120)

study. First, we determined whether the subjects met the Hardy-Weinberg equilibrium using a chi-squared test. For the case and control cohorts, the χ^2 values were 4.85 and 0.14, respectively (both $p > 0.05$). Therefore, the selected samples represent the whole population. The average ages of the control group and the breast cancer group were 43.79 ± 9.78 and 47.43 ± 10.29 years, respectively, and there was no significant difference between the ages of these two groups ($p = 0.340$). We did not find any other statistically difference between the characteristics of the two groups.

Next, we analyzed the genotype distribution of the rs17251221 polymorphism using a TaqMan allelic discrimination assay. As shown in Table 1, we found higher proportions of the AG and GG genotypes in the cancer cases than in the control group (AG: 21.66% vs. 14.72%; GG: 4.15% vs. 0.43%, respectively). Logistic regression analysis indicated that there were significantly different distributions of the genotypes between the two groups ($p = 0.037$ and $p = 0.007$, respectively). Additionally, the GG and AG genotypes were associated with an increased risk of breast cancer compared to the AA genotype (OR=1.683, 95% CI=1.033-2.742; and OR=10.957, 95% CI=1.374-87.393, respectively). As there were only 9 cancer patients and 1 control patient with the GG genotype, we combined the GG and AG genotypes in the following analyses. We found that the frequency of the G allele was higher in the cancer patients (25.81%) than in the controls (15.15%). The analysis results showed that patients who had a G allele had a 1.948-fold higher risk for breast cancer than patients with an A allele

Table 2. Association between rs17251221 and clinicopathological variables. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Triple-negative, ER, PR and HER2 negative

Variables	Number	AA	genotype AG+GG	P value
Age				0.006
≤45	102	33(32.35%)	69(67.65%)	
>45	115	59(51.30%)	56(48.70%)	
Pathological type				0.621
Ductal	171	78(45.61%)	93(54.39%)	
others	46	19(41.30%)	27(58.70%)	
Tumor size(cm)				0.011
≤2	132	47(35.61%)	85(64.39%)	
>2	71	39(54.93%)	32(45.07%)	
Histological grade				0.243
Low	79	37(46.84%)	52(53.16%)	
High	40	12(30.00%)	28(70.00%)	
Lymph node metastasis				0.021
No	117	69(58.97%)	48(41.03%)	
Yes	66	27(40.91%)	39(59.09%)	
ER				0.001
Negative	47	35(74.47%)	12(25.53%)	
Positive	96	42(64.58%)	54(35.42%)	
PR				0.193
Negative	70	31(44.29%)	39(55.71%)	
Positive	81	45(55.56%)	36(44.45%)	
HER2				0.380
Negative	108	45(41.67%)	63(58.33%)	
Positive	81	39(48.15%)	42(51.85%)	

Fig. 3. The relationship between the rs17251221 polymorphism and breast cancer prognosis according to Kaplan-Meier analysis. (A) The AG+GG genotypes of rs17251221 had shorter relapse-free survival ($p<0.001$). (B) The AG+GG genotypes of rs17251221 were associated with shorter overall survival ($p=0.015$).

($p=0.007$, OR=1.948, 95% CI=1.216-3.120) (Table 1). Taken together, our finding suggested that the G allele might be a risk factor for the development of breast cancer.

The association between the rs17251221 polymorphism and clinicopathological variables
We first detected the expression of CaSR protein in the clinical samples and found that the percentage of CaSR negative cases was significantly higher in the AG+GG genotypes than in the AA genotype ($p=0.0316$) (Table 2). Additionally, we summarized the association of the rs17251221 genotypes with the clinicopathological characteristics in Table 2, including the patient's age at diagnosis, tumor size, pathological type and histological grade, lymph nodal status, estrogen receptor (ER) status, progesterone receptor (PR) status and human epidermal growth factor receptor 2 (HER2) status. We found that the GG+AG genotypes were associated with the patient's age at diagnosis ($p=0.006$), tumor size ($p=0.011$), lymph node metastasis ($p=0.021$) and ER status ($p=0.001$) in the breast cancer patients, but were not associated with the histological grade, pathological type, PR status, or HER2 status.

The association between the rs17251221 polymorphism and breast cancer prognosis

As we found that the rs17251221 polymorphism was associated with age, tumor size and lymph node metastasis, these results suggested that the rs17251221 polymorphism may be a prognostic indicator for breast cancer survival. Therefore, we evaluated the rs17251221 genotypes with respect to the disease-free survival and overall survival. As shown in Figure 3, we found that the GG+AG genotypes of the rs17251221 SNP were correlated with a shorter disease-free survival and a shorter overall survival using Kaplan-Meier analysis of sixty-five patients ($p < 0.001$, $p = 0.015$, respectively). We further performed Cox proportional hazards regression analysis to investigate the independent prognostic value of the rs17251221 polymorphism. The patient's age at diagnosis, the size of the primary tumors, lymph node status, ER status, PR status, and HER-2 status were involved in the multi-analysis. However, the GG + AG genotypes of the rs17251221 SNP failed to retain its prognostic value for disease-free survival ($p = 0.114$) and overall survival ($p = 0.698$). These data suggested that this SNP was not an independent prognostic factor.

Discussion

Breast cancer is still one of the most common malignant diseases in the world. Unfortunately, the detailed pathogenesis of breast cancer has not yet been completely elucidated. Genomic polymorphisms are widely dispersed in the human genome, and single nucleotide polymorphisms are one of the most prevalent forms of human genetic variation [18]. Recently, several genome wide association studies have reported many genetic variants that are associated with breast susceptibility [19-23]. Therefore, identifying novel functional single nucleotide polymorphisms is a novel, promising avenue for the prevention of breast cancer.

CaSR is expressed at different lesions, including in normal and fibrocystic breast duct cells, as well as ductal carcinomas of the breast [24]. Several experiments have confirmed that CaSR acts as a tumor suppressor in breast cancer. It has been reported that extracellular Ca^{2+} can down-regulate cell proliferation, invasion and anchorage-independent growth in cancer [25, 26]. Recent studies showed that BRCA1 expression could increase the sensitivity of breast cancers to paclitaxel by regulating CaSR [27-29]. We investigated the rs17251221 SNP because it is one genetic variation of CaSR. Previous studies found that rs17251221 was strongly associated with serum calcium regulation [11], and it has been shown to be a susceptibility marker for stone multiplicity in nephrolithiasis [13]. Therefore, rs17251221 may be a potential functional polymorphism in breast cancer.

To confirm the above hypothesis, we first detected the mRNA levels of CaSR in breast cancer tissues with different genotypes, and we found that the GG+AG genotypes had lower mRNA and protein levels compared to the AA genotype. These data suggested that the polymorphism had an effect on CaSR expression, which may subsequently contribute to the development and progression of breast cancer. We next investigated the role of rs17251221 in breast cancer susceptibility in a Han Chinese population in a case-control study. Our data demonstrated that the GG+AG genotypes were associated with breast cancer susceptibility. Because the ER, PR and HER statuses are well-established indices of survivability, we analyzed the relationship between CaSR and ER, PR and HER-2 in the clinical samples, and found that CaSR was significantly associated with ER status. However, we found that CaSR did not correlate with PR status or HER2 status. This may be due to the small number of cases in our study, and therefore further studies enrolling a larger cohort of cases are needed to determine relationship between CaSR and PR as well as HER2 status in the future. More importantly, we found that the GG+AG genotypes were prognostic indicators for both disease-free survival and overall survival of breast cancer.

To the best of our knowledge, this is the first study to reveal that the rs17251221 polymorphism in CaSR was associated with breast cancer susceptibility and prognosis. However, further investigations are warranted to understand the precise mechanism of this

polymorphism. Our present findings suggest that rs17251221 may play an important role in the development of breast cancer and, therefore, may be a vital prognostic indicator for breast cancer in the future.

Conflicts of Interest

The authors declare that they have no competing interests.

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